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PCT/EP 03/08948

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Patentanmeldung Nr. Patent application No. Demande de brevet n°

02078273.6

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Anmeldung Nr:
Application no.: 02078273.6
Demande no:

Anmeldetag:
Date of filing: 08.08.02
Date de dépôt:

Anmelder/Applicant(s)/Demandeur(s):

Rijk Zwaan Zaadteelt en Zaadhandel B.V.
Burgemeester Crezeelaan 40
2678 KX De Lier
PAYS-BAS

Bezeichnung der Erfindung/Title of the invention/Titre de l'invention:
(Falls die Bezeichnung der Erfindung nicht angegeben ist, siehe Beschreibung.
If no title is shown please refer to the description.
Si aucun titre n'est indiqué se referer à la description.)

Method of chloroplast transformation in asteraceae

In Anspruch genommene Priorität(en) / Priority(ies) claimed /Priorité(s) revendiquée(s)
Staat/Tag/Aktenzeichen/State/Date/File no./Pays/Date/Numéro de dépôt:

Internationale Patentklassifikation/International Patent Classification/
Classification internationale des brevets:

C12N15/00

Am Anmeldetag benannte Vertragstaaten/Contracting states designated at date of filing/Etats contractants désignées lors du dépôt:

AT BE BG CH CY CZ DE DK EE ES FI FR GB GR IE IT LI LU MC NL PT SE SK TR

METHOD OF CHLOROPLAST TRANSFORMATION IN *ASTERACEAE*

Field of the invention

The invention relates to methods of genetically transforming plant plastids, and morespecifically to genetically transforming the plastid genomes of *Asteraceae* plant species.

Background

Plant plastids (e.g. chloroplasts, amyloplasts, elaioplasts, etioplasts, chromoplasts) are organelles in which major biochemial processes (i.e. photosynthesis) take place. In general, plant cells contain between 100-10,000 copies of a small 120-160 kb circular genome. Since each molecule has one inverted repeat it is theoretically possible to obtain plant cells with 20,000 copies of (a) gene(s) of interest, after chloroplast transformation.

The genetic transformation of the plastid genome (plastome) has major advantages over nuclear transformation. Firstly, in most plant species, plastids are maternally inherited, which minimizes out-crossing of transgenes related to weeds or other crops. This form of genetic engineering of plants lowers the risk of dissemination of the transgene in the environment through pollen dispersal. Furthermore, the plastid genome is highly polyploid, enabling the introduction of many copies per cell which can lead to high accumulation levels of the desired protein(s). The fact that chloroplasts are able to form disulfide bands and to fold human proteins, makes this technique ready for the production of biopharmaceuticals in plants.

The principle of chloroplast transformation is transfer of sequences through homologous recmbination. Chloroplast transformation vectors use two targeting vectors

that flank the foreign genes and insert them, at a precise, predetermined position in the chloroplast genome. Position effects and gene silencing, major problems in nuclear transformation experiments, have not as yet been observed in chloroplast transformation events.

Successful chloroplast transformation of crop plants is described thus far only for *Solanaceous* crops like potato, tomato, tobacco (U.S. Patent Number 5.451,513; Svab et al. (1990), Proc. Natl Acad. Sci USA 87:8526-8530) and rapeseed.

Detailed description of the invention

Methods and compositions for an efficient and stable transformation of chloroplasts of a lettuce plant are provided. DNA constructs are provided for stable transformation of plastids of multicellular structures and plants of lettuce.

As explant material, plant mesophyl protoplasts are used and via PEG transformation transplastomic protoplast-derived colonies and regeneration of meristems were obtained. The DNA constructs comprise a transforming DNA which is targeted to a pre-determined location in the plastid genome and inserted into the plastid genome by homologous recombination. The targeting segments comprise preferred sequences of the lettuce DNA chloroplast genome, i.e. the *trn*I(*ori*A)/*trn*A region or the 16S/trnV/ORF70B region of the lettuce chloroplast genome. The DNA used for transformation contains a non-lethal selectable marker gene which confers a selectable phenotype on cells having the plastids with the transforming DNA. Furthermore the transforming DNA comprises at least one additional DNA sequence, such as encoding a green fluorescent protein (gfp). The non-selectable marker coding segment preferred, is the coding region of aadA from bacteria (E. coli) which encodes aminoglycoside 3 "-

adenylyltransferase to confer spectinomycin and streptomycin resistance. The constructs furthermore are provided with a promoter and a terminator sequence functional in plant plastids.

Legend to Figure 1:

A: PCR products of the ATPase gene.
1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
9 and 10 untransformed callus,
11 and 12 pLCV2-MSK18

B: PCR products of the AadA gene.
1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
9 and 10 untransformed callus,
11 and 12 pLCV2-MSK18

C: PCR products of the trnI junction.
1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,

5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
9 untransformed callus

D: PCR products of the trnA junction.

1. Marker,
2. TRSL5-01016 pLCV2-MSK18-1,
3. TRSL5-01016 pLCV2-MSK18-1
4. TRSL5-02002 pLCV2-MSK18-1-1,
5. TRSL5-02002 pLCV2-MSK18-1-2,
6. TRSL5-02002 pLCV2-MSK18-2-1,
7. TRSL5-02002 pLCV2-MSK18-2-1,
8. TRSL5-02002 pLCV2-MSK18-2-2,
9 untransformed callus

EXAMPLES

EXAMPLE 1

Vector constructions

Construction of LCV1

The lettuce chloroplast vector LCV1 consists of 4571 bp of lettuce chloroplast genome sequence with a unique 16 bp *Pac*1/*Asc*1 site added (Figure 1), cloned into *Sac*1/*Kpn*1 restriction sites on the polylinker of a pBluescript SK+ backbone vector (Figure 2). The lettuce sequence spans from the *rps*7/3'-*rps*12 intergenic region to the16SrRNA/*trn*I intergenic region and corresponds to nucleotide postions 100021-104387 in the tobacco chloroplast genome (GI accession number Z00044). An alignment of this lettuce sequence with the tobacco chloroplast genome sequence is given in Figure 3. The following description of the construction of LCV1 is outlined in Figure 4.

Four primers LCV1A, LCV1B, LCV1C and LCV1D were used to amplify this region in two halves (LCV1A-B and LCV1C-D) and to introduce a unique *Pac*1/*Asc*1 restriction site in the ORF70B/*trn*V intergenic region at the position corresponding to nt 102367 in the tobacco chloroplast genome sequence. DNA from clone 6 of the *Sac*1 library of the lettuce chloroplast genome (Jansen and Palmer, 1987) was used as a template for the LCV1 vector. LCV1A and LCV1B amplified a 2575 bp fragment (2551 bp lettuce sequence + 24 bp extension) LCV1A-B spanning from the *rps*7/3'-*rps*12 intergenic to the ORF70B/*trn*V intergenic region (corresponding to 100021-102367 in the tobacco chloroplast genome). Primer LCV1A contains a *Sac*1 site and LCV1B contains *Pac*1/*Asc*1 sites so that *Sac*1 and *Pac*1/*Asc*1 sites are incorporated at the 5' and 3' end, respectively, of the LCV1A-B fragment. The LCV1 A-B fragment was cloned into the *E.coli* plasmid vector PCR2.1 to create PCR2.1 LCV1A-B. These clones were screened for orientation using *Sac*1 and *Sac*1+*Xba*1. The *Sac*1/*Xba*1 insert was cloned into the polylinker of pBluescript to create pBSLCV1 A-B. Primers LCV1C and LCV1D amplified a 2042 bp fragment (2020 bp lettuce sequence + 22 bp extension) LCV1 C-D. The LCV1C primer contains *Pac*1/*Asc*1 sites and the LCV1D primer contains a *Kpn*1 site so that a *Pac*1/*Asc*1 and a *Kpn*1 site are added to the 5' and 3' end, respectively, of the LCV1 C-D fragment. The LCV1 C-D fragment was cloned into PCR2.1 to create PCR2.1 LCV1 C-D. For the final cloning step, PCR2.1 LCV1 C-D was restricted with *Asc*1+*Kpn*1 to release a 2031 base pair insert that was ligated to pBS A-B, which was linearised with *Asc*1+*Kpn*1, creating LCV1.

Construction of LCV2

LCV2 consists of a 2253 bp lettuce chloroplast genome sequence (Figure 5) spanning from the 16S rRNA/*trn*I

intergenic region to the *trn*A/23S rRNA intergenic region, cloned into the PCR2.1 (Invitrogen) backbone vector (Figure 6). This sequence corresponds to nucleotide positions 104366-106260 in the tobacco chloroplast genome (GI accession number Z00044). An alignment of this lettuce sequence with the tobacco chloroplast genome sequence is given in (Figure 7). The following description of the construction of LCV2 is outlined in Figure 8. Four primers LCV2A, LCV2B, LCV2C and LCV2D were used to amplify this region in two halves (LCV2A-B and LCV2C-D) and to introduce unique *Pac*1/*Asc*1 restriction sites in the intergenic region between the *trn*I and *trn*A genes at the position corresponding to nucleotide 105370 in the tobacco chloroplast genome. For the first half (A-B) of the vector, DNA from clone 6 of the *Sac*1 library of the lettuce chloroplast genome (Jansen and Palmer, 1987) was used as a template. Primers LCV2A and LCV2B amplified a 1258 bp fragment (1242 bp lettuce sequence + 16 bp extension) (LCV2A-B) spanning from 16SrRNA/*trn*I intergenic region to the *trn*I/*trn*A intergenic region. This fragment was cloned into the *E. coli* plasmid cloning vector PCR2.1 (Invitrogen) to create PCR2.1 LCV2A-B. Primer LCV2B contains *Pac*1/*Asc*1 sites so that the LCV2A-B fragment has *Pac*1/*Asc*1 sites at the 3'end. PCR2.1 LCV2 A-B clones were screened for orientation by digestion with *Kpn*1/*Asc*1, which releases a fragment of approximately 1300 bp, and *Xba*1/*Asc*1 which linearised clones with the correct orientation for subsequent cloning. For the second half of the vector chloroplast DNA from lettuce cultivar Evola (Leen de Moss seeds) was used as a template because the entire *trn*A gene was not contained in a single clone in the lettuce chloroplast genome library. Primers LCV2C and LCV2D amplified a 1011 bp fragment (995 bp lettuce sequence + 16 bp extension) LCV2C-D. This sequence spans from the *trn*I/*trn*A intergenic region to the *trn*A/23S rRNA

intergenic region. Primer LCV2C contains *Pac*1/*Asc*1 sites so the fragment LCV2C-D has *Pac*1/*Asc*1 sites at its 5′ end. This fragment was cloned into PCR2.1 to create PCR2.1 LCV2 C-D. These clones were screened for orientation using *Kpn*1+*Asc*1, which linearises clones with required orientation and *Xba*1+*Asc*1, which releases a fragment of approximately 1000 bp in clones with the required orientation. To generate LCV2, the 1.3 kb *Asc*1+*Xba*1 insert from PCR2.1 LCV2C-D was subcloned into PCR2.1 LCV2A-B linearised with *Asc*1+*Xba*1.

Construction of LCV1-MSK18 and LCV2 MSK18

MSK18 is an expression cassette adapted from pMSK18 (Hibberd *et al.* 1998). Plasmid MSK18 was a gift from John Gray (Dept. Plant Sciences, University of Cambridge, Downing Street, Cambridge CB2 3EA, UK). Full details of the construction of pMSK18 have been described previously (Hibberd *et al.* 1998). The MSK18 expression cassette consists of the mGFP coding region (Haselhoff *et al.* 1997) fused to a bacterial *trc* promoter (Amman and Brosius, 1985), and an *aad*A coding region, derived from pUC-atpX-AAD (Goldschmidt-Clermont, 1991) fused to a tobacco *rrn* promoter derived from pZS197 (Svab and Maliga, 1993). A tobacco *psb*A 3′ UTR derived from pSZ197 (Svab and Maliga, 1993) is fused to the 3′ end of the *aad*A gene (Figure 9). Using pMSK18 as a template, *Pac*1 and *Asc*1 sites were added by PCR amplifying the cassette with primers containing *Pac*1 (5′) and *Asc*1 (3′) restriction sites to 5′ and 3′ ends of the of the MSK18 expression cassette. The primers used for this were MSK18 A (Forward) 5′-tagttaattaaTTGACAATTAATCATCCGGCTCGT-3′ and MSK18 B (Reverse) 5′-tagggcgcgccTCGAATATAGCTCTTCTTTCTTA-3′. The MSK18 A-B PCR product was cloned into PCR2.1 to create PCR2.1 MSK18. PCR2.1 MSK18 was restricted with *Pac*1/*Asc*1 to release the MSK18 insert that was cloned into the *Pac*1/*Asc*1 sites in

LCV1 and LCV2 to create LCV1-MSK18 (Figure 9) and LCV2-MSK18 (Figure 10).

Molecular characterisation of putative LCV2 transplastomic callus

Total genomic DNA was extracted from the putatively transformed spectinomycin resistant LCV2-MSK18 callus from seven independent transformation events designated 1, 2, 3, B, D, E and G. PCR analysis of this DNA was used to confirm integration of the LCV2-MSK18 sequence into the lettuce chloroplast genome using a combination of primers (Figure 11). To eliminate the possibility of amplification of unintegrated LCV2-MSK18 plasmid DNA, primers P1 and P4 were designed from lettuce chloroplast sequences external to the vector target region. PCR was carried out on DNA isolated from all 6 putatively transformed calli. In all cases, P1 and P4 give two PCR products, a 2476 bp band corresponding to the expected size of a product amplified from an untransformed wild-type chloroplast genome, and a 4623 bp band corresponding to the size of a PCR product expected from a transformed chloroplast genome (Figure 12). For further confirmation of integration, the left and right integration junctions were amplified by PCR using primer pairs P1+P2 and P3+P4. Primers P2 and P3 correspond to the *trc* promoter and the *aadA* gene on the MSK18 cassette sequence. As expected, PCR amplification with primers P1+P2 and P3+P4 produced products of the expected size (1415 bp and 2006 bp) (Figure 12). The PCR products from callus sample B were cloned into PCR2.1 and sequenced using M13 forward and M13 reverse primers. These sequences confirmed that LCV2-MSK18 was integrated in the lettuce chloroplast genome (Figure 13).

EXAMPLE 2

Obtaining seedlings and an *in vitro* stock of plants

Protoplasts of plants are isolated from leaf material of donor plants. In this example the obtaining of leaf shoot cultures is given.

Seed is being sterilized by subsequent washing in 70% ethanol, 0.7% NaOCl solution during 20 minutes and three times washing with sterile demineralized water.

Seeds are sown on Murashige and Skoog (Murashige and Skoog, Physiol. Plant., 15: 473-497, 1962) medium with saccharose 2%, without hormones. Preferentially, seeds can be cultured at 15°C for 2 days in the dark, after which the seeds are transferred to 25°C in the light (3000 lux, photoperiod 16 hr light/8 hr dark). When first true leaves appear, shoot tips are transferred to Murashige and Skoog based medium with 3% saccharose, without hormones. These sterile shoot cultures are grown under similar growth conditions.

EXAMPLE 3

Isolation of protoplasts

Three week old shoot cultures are used for isolation of protoplasts. Leaves are cut into small pieces and preplasmolysed during 1 hr in the dark in PG solution (54.66 g/l sorbitol and 7.35 g/l $CaCL_2.2H_2O$). The PG solution is then replaced by an enzyme solution with 1% celllulase and 0.25% macerozym. Incubation takes place during 16 hrs in the dark at 25°C.

Subsequently, the suspension is filtered through a nylon mesh filter (41µm) en washed with a third of a volume of CPW16S solution (Frearson, E.M., Power, J.B. & Cocking, E.S. (1973), Developmental Biology 33:130-137) by centrifugation at 700 rpm during 8 minutes. In this way, intact protoplasts are collected on the surface of the

supernatant. Protoplasts are washed in W5 solution (9 g/l NaCl, 18.38 g/l CaCl2.2H2O, 0.37 g/l KCL, 0.99 g/l glucose, 0.1 g/l Morpholinoethanesulfonide buffer) by centrifugation at 600 rpm during 5 minutes. With the procedure described, a protoplast yield of approximately 10-15 x 10^6 protoplasts per gram leaf material.

EXAMPLE 4

Selection of protoplast derived calli on spectinomycin resistance

Protoplasts of lettuce, derived as described in example 3, are diluted in culture medium ½ B5 (Gamborg et al. (1968) Exp. Cell Res. 50:151): 375 mg/l $CaCl_2.2H_2O$, 18.35 mg/l NaFeEDTA, 270 mg/l sodium succinate, 103 g/l saccharose, 0.1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) and 0.3 mg/l 6-benzylaminopurin (BAP) and set to a culture density of 6 x 10^4 protoplasts per ml.

The protoplast suspension is mixed 1:1 with ½ B5 culture medium with agarose. The agarose beads are plated in larger petridishes with liquid ½ B5 culture medium on top of it.

The petri dishes are taped with parafilm and cultured at 25°C. One week after initiation of culture the culture medium is diluted with fresh liquid ½ B5 culture medium.

When calli are about 0.5 mm in size they are transferred to callusgrowth medium SH2 (Schenk R.U. and Hildebrandt, A.C. (1972) Can. J. Bot. 50:199-204) with 30 g/l saccharose, 5 g/l agarose, 0.1 mg/l 1-naphtalene acetic acid (NAA) and 0.1 mg/l benzylaminopurin (BAP), and the selecting agent spectinomycin dihydrochloride at concentrations of 0-1000 mg/l. It was found that the optimal concentration of selection is 500 mg/l. The non-resistant calli will appear as

white calli. They also will grow slower as compared to the control calli.

EXAMPLE 5

Transformation of protoplasts with polyethylene glycol and selection for aadA encoded antibiotic resistance

Protoplasts of lettuce, derived as described in example , are set to a density of approximately 1-1.5 x 10^6 protoplasts/0.4-0.6 ml in transformation buffer (0.4 M mannitol, 15 mM $MgCl_2$, 1% (w/v) Morpholinoethane sulfonate (MES), pH 5.8). Subsequently, 10 µl of plasmid suspension (1 µg DNA/µl sterile H_2O) is added to the protoplasts as well as 0.4-0.6 ml PEG solution (40% w/v PEG 6000, 2.36 g/l $Ca(NO_3)_2.4H_2O$ and 7.28 g/l mannitol). Incubation is performed at room temperature for 5-30 minutes. Protoplasts are washed and resuspended in culture medium ½ B5 (Gamborg et al. (1968) Exp. Cell Res. 50:151): 375 mg/l $CaCl_2.2H_2O$, 18.35 mg/l NaFeEDTA, 270 mg/l sodiumsuccinate, 103 g/l saccharose, 0.1 mg/l 2,4 dichlorophenoxyacetic acid (2,4-D) and 0.3 mg/l 6-benzylaminopurin (BAP).

The protoplast suspension is mixed 1:1 with ½ B5 culture medium with agarose. The agarose beads are plated in larger petridishes with liquid ½ B5 culture medium on top of it.

The petri dishes are taped with parafilm and cultured at 25°C. After 6 days selection of the microcalli is performed by adding 500 mg/l of the selective agent spectinomycin dihydrochloride. One week after initiation of culture the culture medium is diluted with fresh liquid ½ B5 culture medium, with addition of spectinomycin hydrochloride. When calli are about 0.5 mm in size they are transferred to callusgrowth medium SH2 (Schenk R.U. and Hildebrandt, A.C. (1972) Can. J. Bot. 50:199-204) with 30 g/l saccharose, 5 g/l

agarose, 0.1 mg/l 1-naphtalene acetic acid (NAA) and 0.1 mg/l benzylaminopurin (BAP), and the selecting agent spectinomycin dihydrochloride at concentrations described above. After 2 weeks calli can be transferred to regeneration medium SHreg (Schenk R.U. and Hildebrandt, A.C. (1972) Can. J. Bot. 50:199-204) with 15 g/l saccharose, 15 g/l maltose, 0.1 mg/l NAA and 0.1 mg/l BAP and spectinomycin dihydrochloride in concentrations desribed above. Spectinomycin resistant calli will appear as green calli amongst white (non-resistant) calli.

Regenerating plants will appear after approximately 6 weeks and furtheron, and can be transferred to rooting medium (Schenk and Hildebrandt, supra) with 30 g/l saccharose and 8 g/l agar with the concentrations of spectinomycin dihydrochloride mentioned above. Alternatively, in transformation vectors where gfp (green fluorescent protein) is added as favourable gene apart from aadA, gfp fluorescence can be detected using an inverted microscope with the proper filter combinations.

Spectinomycin resistant calli were obtained after transformation of protoplasts with the plasmid pLCV2-MSK18. Approximately 40-50% of the protoplast will survive the PEG treatment. Three independent experiments yielded in total 7 resistant calli (see Table 1). These green calli were detected 4-5 weeks after initiation of each experiment. Callus lines of each individual event are maintained on medium SHreg wit the selective agent spectinomycin dihydrochloride.

For pLCV1-MSK18, no resistant calli were detected as yet.

Spectinomyin resistance of plant cells may be the result, apart from transformation with the vector LCV2-MSK18, of spontaneous mutation of chloroplast DNA or insertion of

the DNA into the nucelar genome. Therefore, the callus was screened for the integration of the right and left homologous border segment (see example 9). Additionally, it was determined whether the aadA gene and the gfp gene was correctly integrated in the chloroplast DNA.

The transgenic callus has been obtained using vectors with specific lettuce chloroplast DNA homologous sequences. Selection of transformed cells with the non-lethal selective agent spectinomycin has been succesful. The optimal transformation frequency for lettuce, determined as the number of green calli to the number of surviving protoplasts is about 1 in 6.10^5 protoplasts (Table 1, Exp 1 and 3).

Table 1. Selection of plastid transformants

Treatment	# pps treated	# green calli	# green calli showing regeneration
Exp 1			
Control	None	0	0
Control + PEG	1.26×10^6	0	0
pLCV1-MSK18	1.26×10^6	0	0
pLCV2-MSK18	1.26×10^6	1	0
Exp 2			
Control	None	0	0
Control + PEG	1.20×10^6	0	0
PLCV2-MSK18	2.40×10^6	1	0
Exp 3			
Control	None	0	0
Control + PEG	1.20×10^6	0	0
PLCV2-MSK18	4.80×10^6	5	2

EXAMPLE 6

Transformation of protoplasts via electroporation and selection on aadA encoded antibiotic resistance

Protoplasts, derived as described in example 3, are

suspended in transformation buffer HSB (150 mM KCL, 4 mM $CaCl_2.2H_2O$, 10 mM HEPES (pH 7.2), and enough mannitol to osmotically balance the protoplasts. This is dependent on the genotype but it can easily be found out experimentally. Aliquots of 1 x 10^6 protoplasts /0.5 ml HSB buffer and mannitol are put into a conical centrifuge tube, and plasmid DNA solution is added. Plasmid DNA concentrations in the transformation buffer should preferably be in the range of 10-100 µg/ml. The protoplast-DNA suspension is transferred to the electroporation chamber and electroporated using a single electric pulse (e.g. 325µF, 300 V) The optimal setting can vary with species and cell type, and should be determined in preliminary experiments. The most efficient parameters are set by finding the pulse settings that result in 50% protoplast death by 24h after the shocks. More details of the method are described by G.W. Bates ((1999), Plant transformation via protoplast electroporation. From: Methods in Molecular Biology Vol 111: Plant cell Culture Protocols, Pp 359-373).

After electroporation, protoplasts culture is performed as described in example 4.

EXAMPLE 7

Adjustment of spectinomycin threshold levels in cotyledons

For the adjustment of the optimal concentration of spectinomycin, for selection of cells with chloroplasts, which are transformed with constructs having the aadA gene as selectible marker, 4 day old cotyledons were plated on media with various concentrations of spectinomycin dihydrochloride. The cotyledons were obtained as described in example 1.

EXAMPLE 8

Transformation of plant material via biolistics and selection for aadA encoded antibiotic resistance

For bombartment of cotyledons, seeds were sown as described in example 1. Alternatively, leaf pieces can be used as explant material for shooting, under similar conditions. In this example, the use of cotyledons is desribed. Cotyledons (3 to 12 days old) are placed with the abaxial side onto MS1 medium (Murashige and Skoog, supra) with 30 g/l saccharose, 8 g/l agar and supplemented with 100-200 mg/l carbenicillin 0.1 mg/l benzylaminopurin (BAP) and 0.1 mg/l 1- naphtalene acetic acid (1-NAA) at pH 5.8. Gold particles (0.6 to 1.6 µm) were prepared for transformation by mixing 50 µl of suspension (60 mg/ml 50% glycerol) with 5µg DNA (1µg/µl H_2O), 50 µl $CaCL_2.2H_2O$ (2.5 M) and 20 µl spermidine (0.1 M base). The particle-DNA mixture was incubated at room temperature for 1-3 minutes and centrifuged for 3-10 sec. in an Eppendorf centrifuge. After removal of the supernatant, the coated particles are washed and diluted in 48-60 µl ethanol. The particles (6-8 µl per carrier) are applied to the macrocarrier holders and the bombartment is performed with PDS-1000/He Biolistic particle delivery system (BioRad). Details of the procedure has been described by Klein et al. (Bio/Technology 6: 559-563 1988).

Two to fourteen days after bombartment, the cotyledons are transferred to MS1 medium as described above with the addition of a selective agent (e.g. spectinomycin dhydrochloride at concentration of 500 mg/l). Cultures are transferred onto fresh medium every 2 weeks. When green callus or shoots appear, they are transferred to medium MS2 without carbenicillin, but including the selective agent spectinomycin dihydrochloride.

EXAMPLE 9

Molecular analysis of spectinomycin resistant calli of lettuce

Spectinomycin resistant callus of lettuce was analysed by PCR using different primer combinations to confirm the integration of the plasmid pLCV2-MSK18 in the genome of the chloroplast.

As an endogenous control for chloroplast DNA amplification, PCR analysis of the ATPase gene (Accession: AF162208) was carried out using the forward primer 5'-ACTAATAGTGGACAAATTGGC-3' and the reverse primer 5'-TTGCTTGATTGTATTTACTCG-3'. To detect the presence of the selectable marker gene AadA, the following primer combination was used: forward 5'-TATGACGGGCTGATACTGGGC-3' and reverse 5'-AAGTCACCATTGTTGTGCACG-3'. In order to demonstrate the physical integration of the plasmid into the chloroplast genome 2 primer combinations were developed which amplify hybrid regions of the plasmid and the chloroplast genome. The first primer combination consisting of forward 5'-ACTGGAAGGTGCGGCTGGAT-3' and reverse 5'-ACGAGCCGGATGATTAATTGTCAATTAATTAACTA-3' amplifies the junction containing the trnI sequence of the chloroplast genome. The second primer combination consisting of forward 5'-AAGTCACCATTGTTGTGCACG -3' and reverse 5'-CTCGCCCTTAATTTTAAGGC-3' amplifies the junction containing the trnA sequence of the chloroplast genome.

Total DNA was isolated from spectinomycin resistant callus using a commercially available DNA isolation kit from Sigma (Genelute Plant Genome DNA Kit). The PCR reaction was carried out using a total amount of 30 ng DNA after which the reaction products were analysed on a 1% agarose gel. The result of the analysis of 5 independent spectinomycin resistant calli is shown in Figure 1. The ATPase fragment of

about 424 bp is only present in callus material and leaf material of lettuce, and as expected not visible for the pLCV MSK18 DNA. PCR amplification of the aadA gene gave the expected fragment of approximately 413 bp for the transgenic callus and the plasmid pLCV2-MSK18. To confirm the integration of the pLCV2-MSK18 vector into the lettuce chloroplast genome, the two primer combinations were used which specifically detect either one of the two juntions which emerge after integration of the plasmid by homologous recombination. Figure 1 D shows the amplification of the trnAjunction which results in an expected band of approx. 1500 bp in the spectinomycin resistant callus. The integration on trnI junction was investigated using the PC indicated above which resulted in an expected band of approximately 1500 bp as well. The results of this analysis confirm the transplastomic nature of the obtained spectinomycin resistant letuce calli.

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CLAIMS

1. Method for the transfromation of plant plastids as described in the description.

2. Compositions for use in the method as claimed in claim 1.

Figure 1. LCV1 lettuce chloroplast genome target sequence (not including backbone vector).

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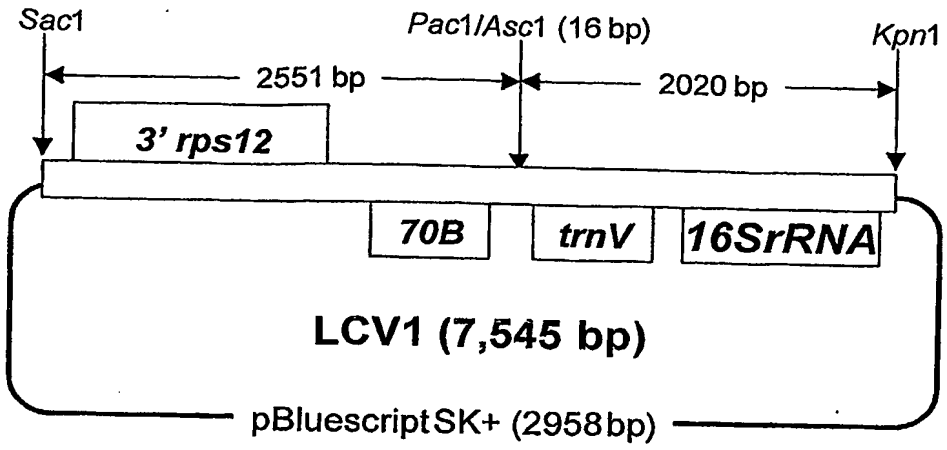

Figure 2. Map of LCV1 (7,535 bp).

Figure 3. LCV1 lettuce chloroplast genome target sequence aligned with tobacco chloroplast genome (GI Z00044).

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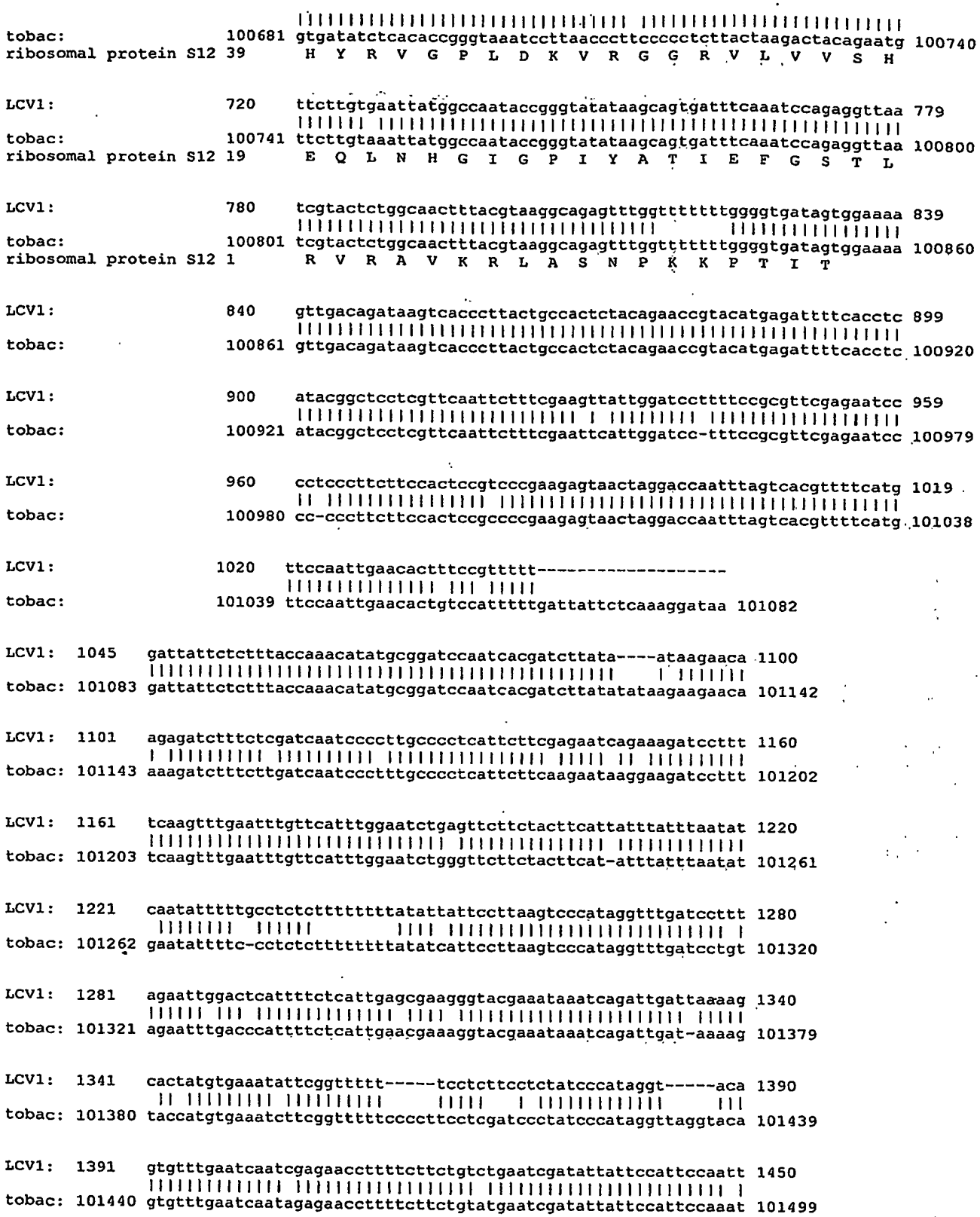
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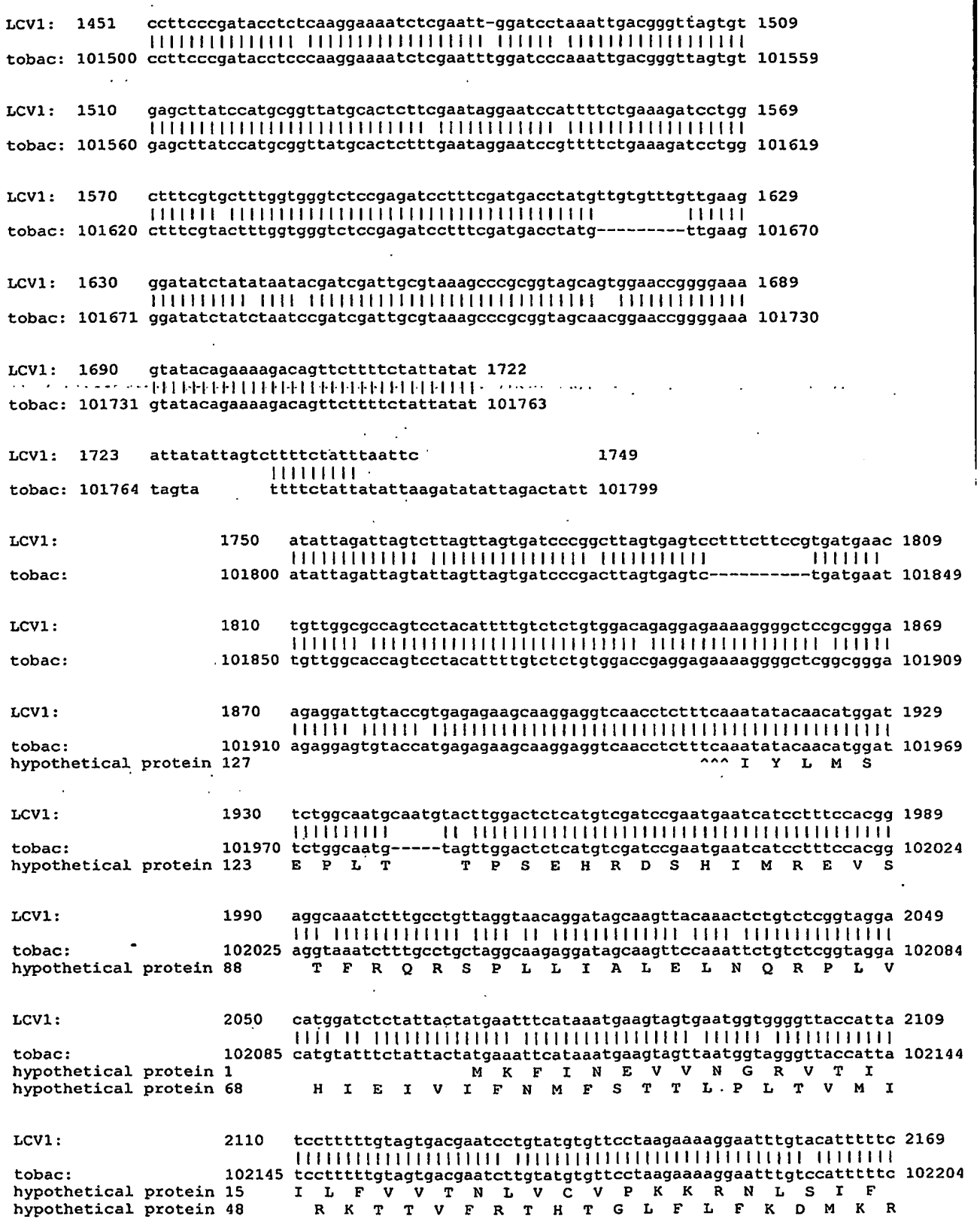
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Tobac:                     ------------------------------------------------


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hypothetical protein 1        M  I  K  M


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LCV1:  2748   gttttgatattttgatttgctaccccgccgtgattgaatgagaatggataagaggctcgt 2807
               |||||  ||  ||  ||||||||  |||||||||  ||  ||||||||||||||||||||||||
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LCV1:  2808   gggattgacgtgaggggcagggatggctatatttctgggagcgaactccgggcgaatat 2867
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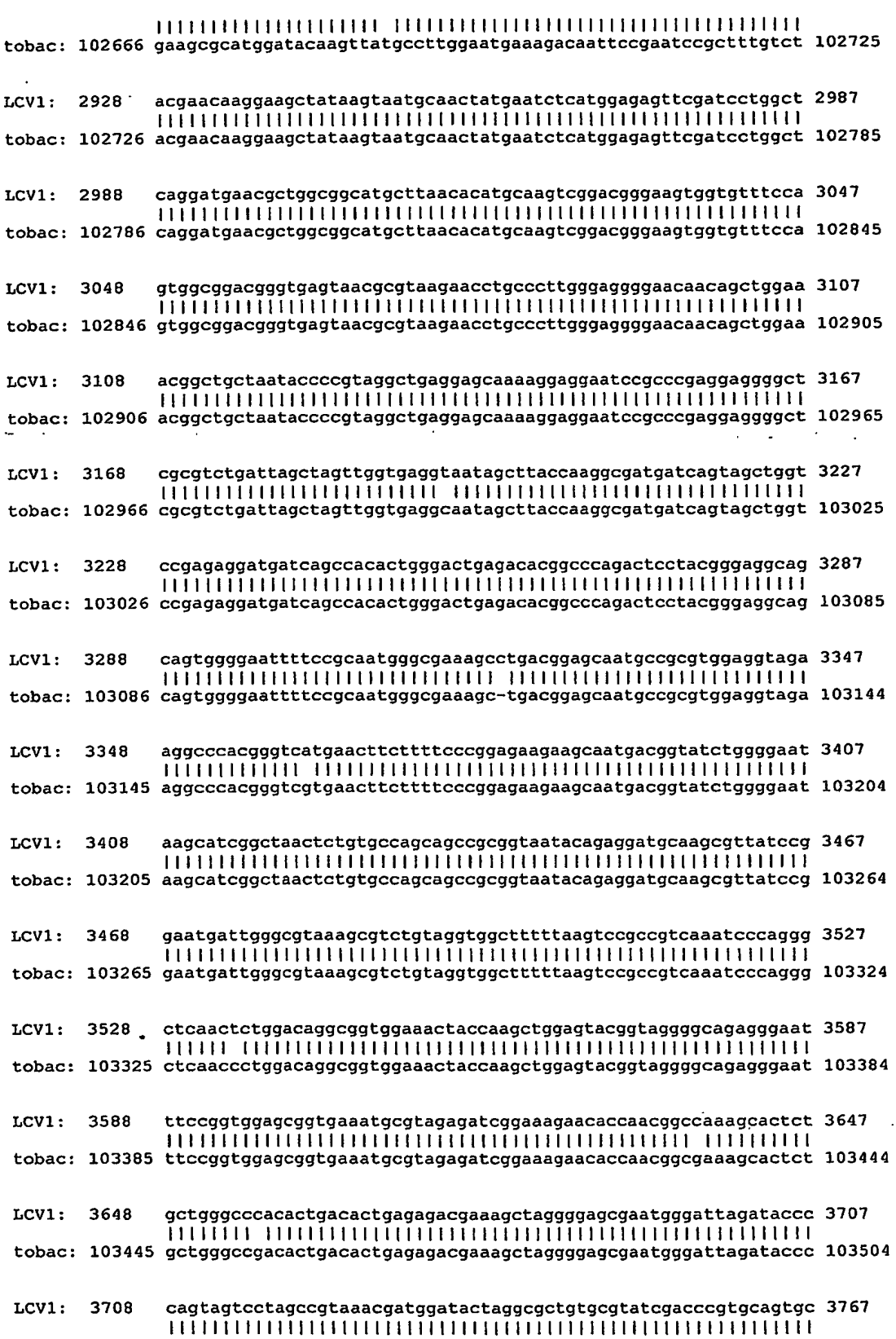

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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tobac: 103565 tgtagctaacgcgttaagtatcccgcctggggagtacgttcgcaagaatgaaactcaaag 103624

LCV1:  3828   gaattgacgggggcccgcacaagcggtggagcatgtggtttaattcgatgcaaagcgaag 3887
              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
tobac: 103625 gaattgacgggggcccgcacaagcggtggagcatgtggtttaattcgatgcaaagcgaag 103684

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LCV1:  4068   ctgccggtgataagccggaggaaggtgaggatgacgtcaagtcatcatgcccctttatgcc 4127
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LCV1:  4128   ctgggcgacacacgtgctacaatggccgggacaaagggtcgcgatcccgcgagggtgagc 4187
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              ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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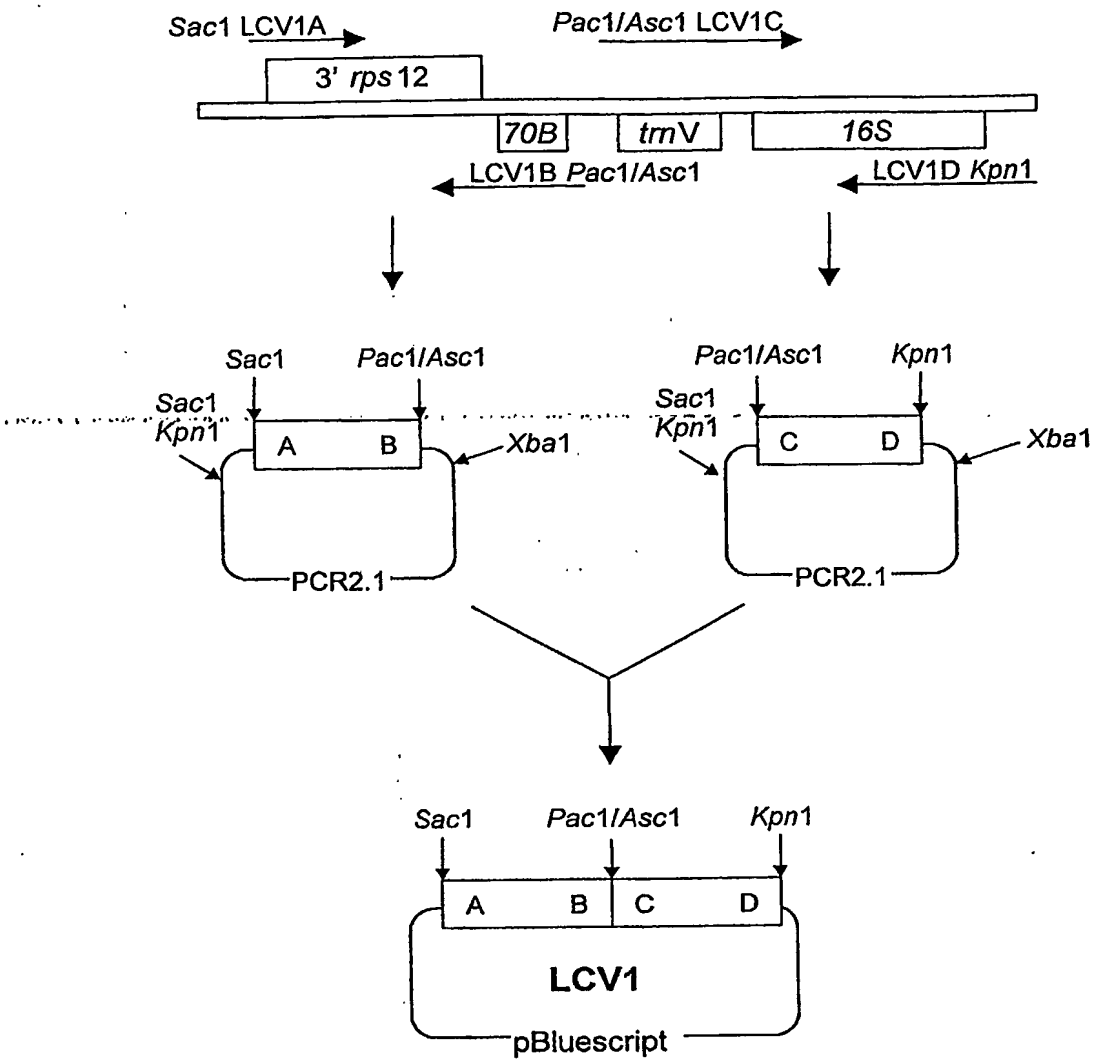

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LCV1C-5'TTAATTAAGGCGCGCCAGGCCCGGCCCCAAGTT3' (102368-102384 in TCG)
LCV1D-5'ATGGTACCCTTGGTCTTACTTCACTGTCGA3' (104366-104387 in TCG)

Figure 4. Cloning steps and primers for construction of LCV1. TCG = tobacco chloroplast genome.

Figure 5. LCV2 lettuce chloroplast genome target sequence (not including backbone vector).

TCGACAGTGAAGTAAGACCAAGCTCATGAGCTTATTATCTCAGGTCGGAACAAGTTGATAGGATCCCCCTTTTT
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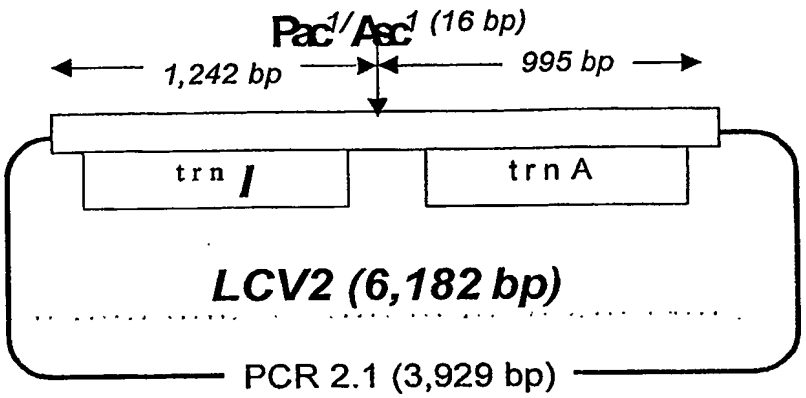

Figure 6. Map of LCV2 (6,182 bp).

Figure 7. LCV2 lettuce chloroplast genome target sequence alignmened with tobacc chloroplast genome (GI Z00044).

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LCV2 : 61      ggatccccctttttacgtccccatg--ccccctgtgtggcgacatgggggcgaaaaaagg 118
                ||| |||| ||||||||||||||||  ||||| ||||||||||||||||||||||||||
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LCV2 : 119     aaagagagagatggggtttctctcgcttttggcatagtgggcccccagtgggggctcgc 178
                |||||||| |||||||||||||||||||||||||||||| |||||||||||||| |||||||
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                ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
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LCV2 : 299     gatgtggatcatccaaggcacattagcatggcgtactcctcctgttcgaaccggggtttg 358
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LCV2 : 359     aaaccaaacttctcctcaggaggatagatggggcgattcaggtgagatccaatgtagatc 418
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LCV2 : 419     caactttcgattcactcgtgggatccgggcggtccgggggggaccaccatggctcctctc 478
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gtataaataactcacttcttggtcttcgacccccgcagtcactacgaacgcccccgatcagtgcaatgggatgtgtctatttatctatc
895
(231 bp present in lettuce maize, rice and soybean but not tobacco)
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```
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tobac: 106203 ttatgatagcacccaattttccgattcggcggttcgatctatgatttatcattcatg 106260
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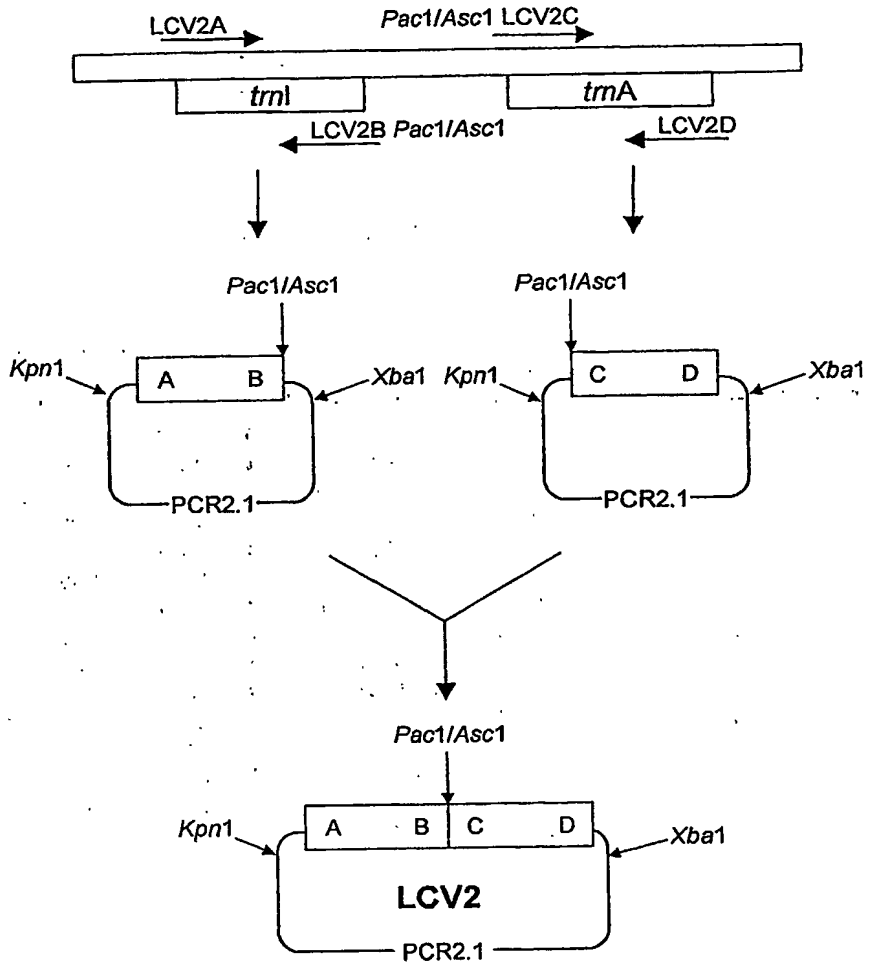


LCV2A 5′TCGACAGTGAAGTAAGACCAAG3′ (104366-104387 in TCG)
LCV2B 5′GGCGCGCCTTAATTAAGGAGTCAGACGCTTCTTCTATTC3′ (10346-105370 in TCG)
LCV2C 5′TTAATTAAGGCGCGCCCATGCATGCTCCACTTGGCTCGG3′(105371-105393 in TCG)
LCV2D 5′CATGAATGATAAATCATAGATCGAAC3′ (106234-106260 in TCG)

Figure 8. Cloning steps and primers for construction of LCV2. TCG= tobacco chloroplast genome.

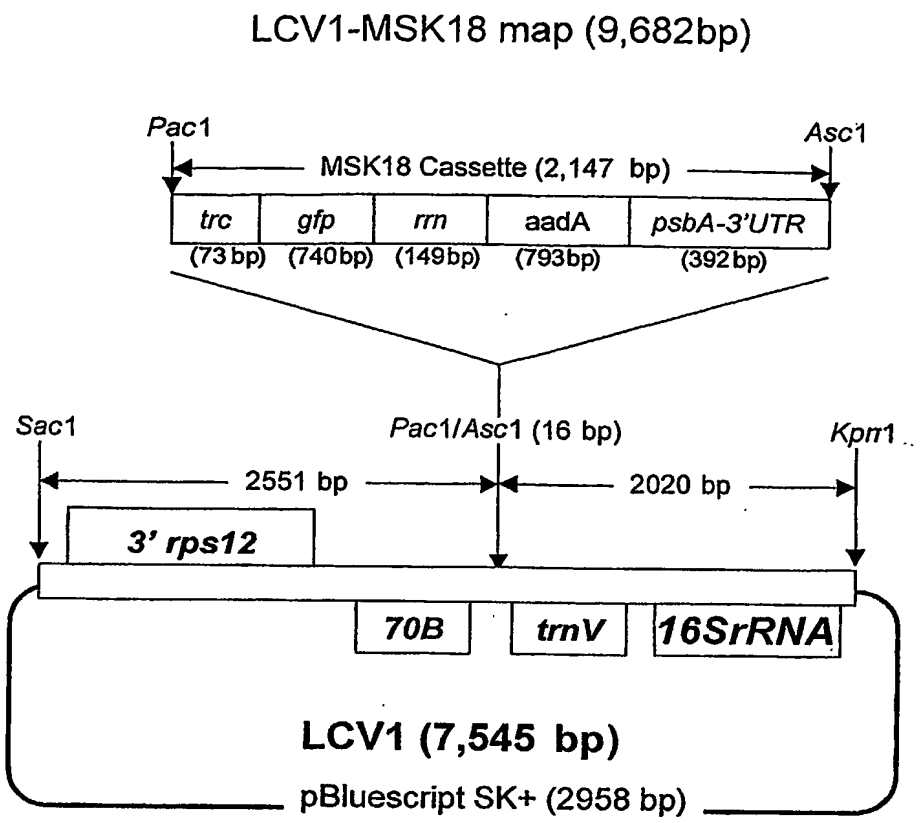


Figure 9. Map of LCV1 MSK18 (9,692 bp).

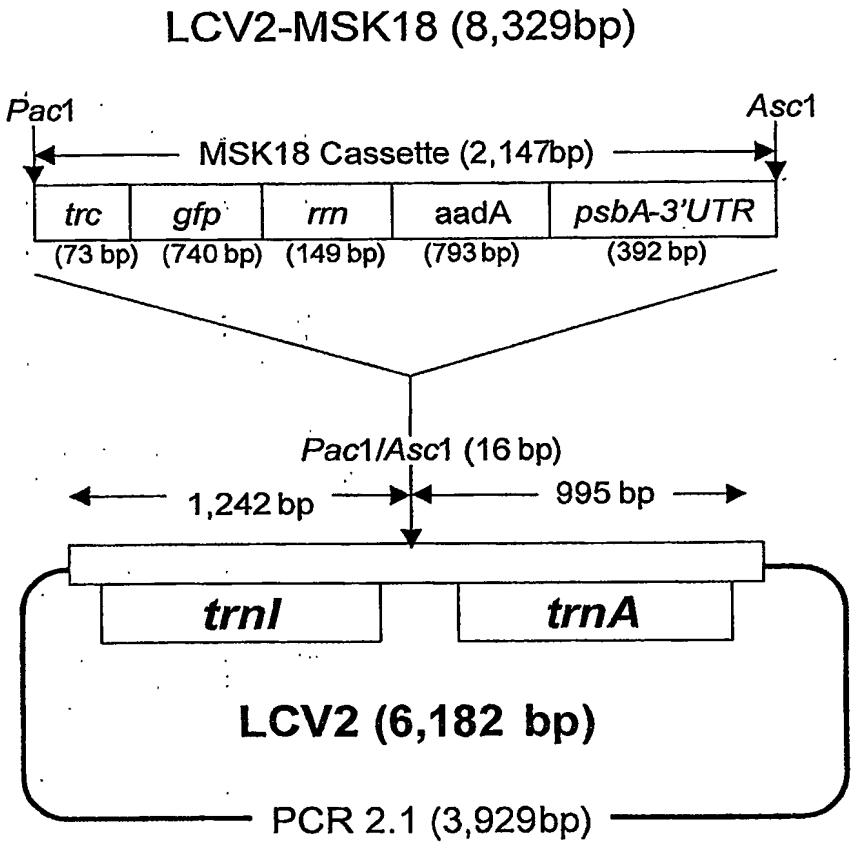
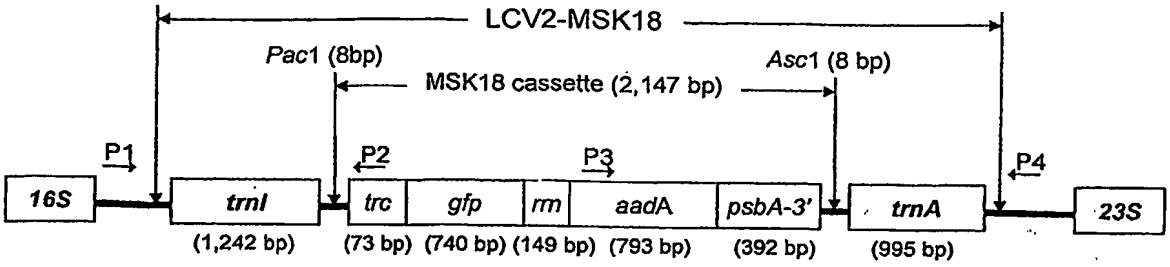


Figure 10. Map of LCV2-MSK18 (8,329 bp).



P1 + P2 = 1415 bp
P3 + P4 = 2006 bp
P1 + P4 = 4623 bp

P1 5′-ACTGGAAGGTGCGGCTGGAT-3′
P2 5'-ACGAGCCGGATGATTAATTGTCAATTAATTAACTA-3' (MSK18A comp)-
P3 5'-AAGTCACCATTGTTGTGCACG-3′ (starts at 259 bp on *aadA* CDS)
P4 5′-CTCGCCCTTAATTTTAAGGC-3′

Figure 11. Primer combinations used in PCR analysis of transplastomic lettuce callus.

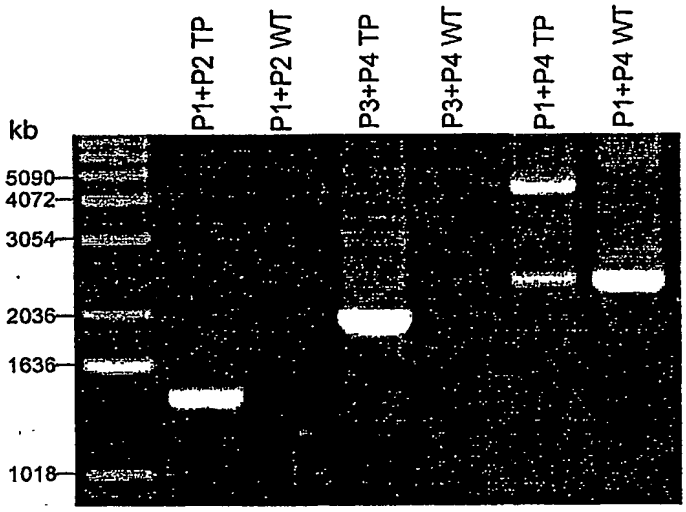


Figure 12. Agarose gel electrophoresis of PCR products from reactions with primer pairs P1+P2, P3+P4 and P1+P4 and template DNA from spectinomycin resistant putative transplastomic callus sample B (TP) and non-transformed wild-type callus (WT).

Figure 13. Sequence of left border (P1-P2) and right border (P3-P6) integration junction fragments amplified by PCR from transplastomic lettuce DNA. Sequence in lower case is lettuce chloroplast DNA external to the LCV2 vector target region.

P1-P2 left border fragment consensus sequence

Primer P1→

actggaaggtgcggctggatcacctccttttcagggagagctaatgcttgttgggtattttggtttgacac
tgcttcacacccaaaaaagaagggagctacgtctgagttaaacttggagatggaagtcttcatttcgtttc
Primer LCV2A→=LCV2A left border
TCGACAGTGAAGTAAGACCAAGCTCATGAGCTTATTATCTCAGGTCGGAACAAGTTGATAGGATCCCCCTT
TTTACGTCCCCATGCCCCCTGTGTGGCGACATGGGGGCGAAAAAAGGAAAGAGAGAGATGGGGTTTCTCTC
GCTTTTGGCATAGTGGGCCCCCAGTGGGGGGCTCGCACGACGGGCTATTAGCTCAGTGGGTAGAGCGCGCC
CCTGATAATTGCGTCGTTGTGCCTGGGCTGTGAGGGCTCTCAGCCACATGGATAGTTCAATGTGCTCATCG
GCGCCTGACCCTGAGATGTGGATCATCCAAGGCACATTAGCATGGCGTACTCCTCCTGTTCGAACCGGGGT
TTGAAACCAAACTTCTCCTCAGGAGGATAGATGGGGCGATTCAGGTGAGATCCAATGTAGATCCAACTTTC
GATTCACTCGTGGGATCCGGGCGGTCCGGGGGGGACCACCATGGCTCCTCTCTTCTCGAGAATCCATACAT
CCCTTATCAGTGTATGGACAGCTATCTCTCGAGCACAGGTTTAGGTTCGGCCTCAATGGGAAAATAAAATG
GAGCACCTAACAACGCATCTTCACAGACCAAGAACTACGAGATCACCCCTTTCATTCTGGGGTGACGGAGG
GATCATACCATTCGAGCCTTTTTTTTTCATGCTTTTCCCCGAGGTCTGGAGAAAGCTGAAATCAATAGGAT
TTCCCTAATCCTCCCTTACCGAAAGGAAGAGCGTGAAATTCTTTTTCCTTTCCGCAGGGACCAGGAGATTG
GATCTAGCCGTAAGAAGAATGCTTGGTATAAATAACTCACTTCTTGGTCTTCGACCCCCGCAGTCACTACG
AACGCCCCCGATCAGTGCAATGGGATGTGTCTATTTATCTATCTCTTGACTCGAAATGGGAGCAGGTTTGA
AAAAGGATCTTAGAGTGTCTAGGGTTGGGCCAGGAGGGTCTCTTAACGCCTTCTTTTTTCTTCTCATCGGA
GTTATTTCACAAAGACTTGCCATGGTAAGGAAGAAGGGGGGAACAGGCACACTTGGAGAGCGCAGTACAAC
GGAGAGTTGTATGCTGCGTTCGGGAAGGATGAATCGCTCCCGAAAAGGAATCTATTGATTCTCTCCCAATT
GGTTGGACCGTAGGTGCGATGATTTACTTCACGGGCGAGGTCTCTGGTTCAAGTCCAGGATGGCCCAGCTG
Pac1 *trc* promoter→ ←Primer P2
CGCCAGGGAAAAGAATAGAAGAAGCGTCTGACTCC[TTAATTAA][TTGACAATTAATCATCCGGCTCGT]

P3-P6 left border fragment consensus sequence

Primer P3→(*aadA* gene)
AAGTCACCATTGTTGTGCACGACGACATCATTCCGTGGCGTTATCCAGCTAAGCGCGAACTGCAATTTGGA
GAATGGCAGCGCAATGACATTCTTGCAGGTATCTTCGAGCCAGCCACGATCGACATTGATCTGGCTATCTT
GCTGACAAAAGCAAGAGAACATAGCGTTGCCTTGGTAGGTCCAGCGGCGGAGGAACTCTTTGATCCGGTTC
CTGAACAGGATCTATTTGAGGCGCTAAATGAAACCTTAACGCTATGGAACTCGCCGCCCGACTGGGCTGGC
GATGAGCGAAATGTAGTGCTTACGTTGTCCCGCATTTGGTACAGCGCAGTAACCGGCAAAATCGCGCCGAA
GGATGTCGCTGCCGACTGGGCAATGGAGCGCCTGCCGGCCCAGTATCAGCCCGTCATACTTGAAGCTAGAC
AGGCTTATCTTGGACAAGAAGAAGATCGCTTGGCCTCGCGCGCAGATCAGTTGGAAGAATTTGTCCACTAC
aadA stop/*psbA* 3'UTR→
GTGAAAGGCGAGATCACCAAGGTAGTCGGCAAATAATGTCTAGAGCGATCCTGGCCTAGTCTATAGGAGGT
TTTGAAAAGAAAGGAGCAGTAATCATTTTCTTGTTCTATCAAGAGGGTGCTATTGCTCCTTTCTTTTTTTC
TTTTTATTTATTTACTAGTATTTTACTTACATAGACTTTTTTGTTTACATTATAGAAAAAGAAGGAGAGGT
TATTTTCTTGCATTTATTCATGATTGAGTATTCTATTTTGATTTTGTATTTGTTTAAAATTGTAGAAATAG
AACTTGTTTCTCTTCTTGCTAATGTTACTATATCTTTTTGATTTTTTTTTCCAAAAAAAAAATCAAATTTT
GACTTCTTCTTATCTCTTATCTTTGAATATCTCTTATCTTTGAAATAATAATATCATTGAAATAAGAAAGA
Asc1 *trnA* gene→
AGAGCTATATTCGA[GGCGCGCC]CATGCATGCTCCACTTGGCTCGGGGGGATATAGCTCAGTTGGTAGA
GCTCCGCTCTTGCAATTGGGTCGTTGCGATTACGGGTTGGATGTCTAATTGTCCAGGCGGTAATGATAGTA
TCTTGTACCTGAACCGGTGGCTCACTTTTTCTAAGTAATGGGGAAGAGGACCGAAACATGCCACTGAAAGA
CTCTACTGAGACAAAGATGGGCTGTCAAGAACGTCAAGAACGTAGAGGAGGTAGGATGGGCAGTTGGTCAG
ATCTAGTATGGATCGTACATGGACGGTAGTTGGAGTCGGCGGCTCTCCTAGGGTTCCCTTATCGGGGATCC

CTGGGGAAGAGGATCAAGTTGGCCCTTGCGAACAGCTTGATGCACTATCTCCCTTCAACCCTTTGAGCGAA
ATGCGGCAAAAGGAAGGAAAATCCATGGACCGACCCCATCATCTCCACCCCGTAGGAACTACGAGATTACC
CCAAGGACGCCTTCGGCATCCAGGGGTCACGGACCGACCATAGAACCCTGTTCAATAAGTGGAACGCATTA
GCTGTCCGCTCTCAGGTTGGGCAGTAAGGGTCGGAGAAGGGCAATCACTCATTCTTAAAACCAGCGTTCTT
AAGGCCAAAGAGTCGGCGGAAAAGGGGGGAAAGCTCTCCGTTCCTGGTTTCCTGTAGCTGGATCCTCCGGA
ACCACAAGAATCCTTAGTTAGAATGGGATTCCAACTCAGCACCTTTTGAGTGAGATTTTGAGAAGAGTTGC
TCTTTGGAGAGCACAGTACGATGAAAGTTGTAAGCTGTGTTCGGGGGGGAGTTATTGTCTATCGTTGGCCT
CTATGGTAGAATCAGTCGGGGGACCTGAGAGGCGGTGGTTTACCCTGCGGCGGATGTCAGCGGTTCGAGTC
*trn*A end
CGCTTATCTCCAACTCGTGAACTTAGCCGATACAAAGCTATATGACAGCACCCAATTTTTCCGATTTGGCG
←Primer LCV2D = RB of LCV2
gttcgatctatgatttatcattcatggacgttgataagatccatccatttagcagcaccttaggatggcat
←Primer P6
agccttaaaattaagggcgag